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Pratima Karnik

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## A. INTRODUCTION

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes (*MYC*, *ERBB2* and *CCND1*), mutations of *TP53*, and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q (1, 2). Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q), and *KISS1* (1q) (1, 3-5). LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15 (2, 6)

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast (6, 7) and other adult (8-12) and childhood cancers (13-17). More recently, we have mapped two distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis (6). LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor suppressor gene(s) encoded by this region have evaded identification.

Integrin-linked kinase (*ILK*) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways (18). It binds to the cytoplasmic domains of  $\beta 1$  and  $\beta 3$  integrins and mediates the down-stream signaling events in integrin function (19). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (20). In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion and *ILK* mutations cause embryonic lethality and defects in muscle attachment (21). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene in tumor suppression has not been established. We have therefore analyzed the effect of *ILK* expression on the *in vivo* tumor growth and invasion of human mammary carcinoma cells.

## B. BODY:

### 1. Results:

#### ***ILK* Suppresses the Invasive Phenotype of Human Breast Carcinoma Cells**

The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases. Cell migration on vitronectin *in vitro* has been linked to the metastatic capacity of tumor cells *in vivo* (22, 23). To examine the effects of *ILK* expression on breast cancer cell invasion, the ability of vector and *ILK* transfected MDA-MB-435 cells to degrade and invade vitronectin-coated polycarbonate membrane was investigated. As shown in Figure-1A, a significant reduction in invasive potential was noted in the *ILK* expressing clone TR5 (*ILK*) compared to vector transfected MDA-MB-435 cells (VT) (Figure-1A). Cell invasion through membranes coated with vitronectin, is decreased by 60% in MDA-MB-435 cells expressing *ILK* compared to vector transfected MDA-MB-435 cells. In contrast, the two *ILK* variants  $\Delta$  ANK and E359K have no significant effect on cell invasion under identical conditions (Figure-1A). In fact, there is a slight increase in invasive potential of the variant clones ( $\Delta$  ANK and E359K), suggesting a dominant-negative effect, perhaps due to inhibition of endogenous *ILK* in the MDA-MB-435 cells. These results indicate that *ILK* expression abates extracellular matrix invasion of tumor cells *in vitro*, one of the hallmarks of tumorigenicity and transformed cell growth.

Cell adhesion, migration and invasion are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell (20). Because the  $\alpha v\beta 3$  and  $\alpha 5\beta 1$

integrins have been implicated in the regulation of angiogenesis, tumor cell migration, invasion and metastasis, we speculated that *ILK* might regulate cell migration via alteration of the cellular composition of integrins. Using a panel of specific antibodies against these integrins in flow cytometry analysis, we compared integrin expression patterns in relation to the *ILK* expression status. The results are shown in Figure-1B. The *ILK* transfected cells demonstrated a 22% increase in levels of the growth-suppressing integrin  $\alpha 5\beta 1$  and a 31% decrease in levels of the growth-promoting integrin  $\alpha v\beta 3$  compared to the control cells. The changes in levels of  $\alpha v\beta 3$  and  $\alpha 5\beta 1$  expression in *ILK* transfected cells although relatively moderate in comparison to control cells, nonetheless, were highly significant. Collectively, these observations suggest that *ILK* reduces the invasive potential of MDA-MB-435 cells by altering their integrin profiles, which changes their ability to perceive and interact with their extracellular environment.

### ***ILK* suppresses tumor formation and metastasis in nude mice**

In the last progress report, we reported that we have transfected the *ILK* gene into the metastatic breast cancer cell line MDA-MB-435 and have isolated four different clones that express different levels of *ILK* mRNA and protein. We are now testing these cells using a nude mouse metastatic model.

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the cellular growth properties commonly associated with the cellular state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenicity under expression of *ILK* *in vivo* would be a critical test to substantiate the growth suppressor function of *ILK*. The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection, metastasizes in nude mice and closely resembles the course of human breast cancer (24). To investigate whether *ILK* expression affected tumor formation in nude mice, two different *ILK* transfectant clones (TR5-*ILK* and TR3-*ILK*) and two vector controls were inoculated into the subaxillary mammary fat pads of 4-6 week old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. All MDA-MB-435 vector transfectants were already palpable 7 days after injection. Subsequently, the tumors of vector transfectants grew steadily attaining mean volumes of 3.0 cm<sup>3</sup> (mean  $\pm$  s.d.) at 15 weeks (Fig. 2A and B). In contrast, only 2 of 12 mice injected with *ILK* transfectants developed tumors. The tumor growth of *ILK* transfectants was significantly slower than that of control transfectants ( $P < 0.005$ , Fisher variance analysis). At sacrifice, (15 weeks) the *ILK* tumors reached a mean volume of only 0.45 cm<sup>3</sup> (mean  $\pm$  s.d.) which was significantly smaller than control tumors ( $P < 0.001$ , Student's *t*-test). Vector transfected MDA-MB-435 cells developed an average of 12-24 lung metastases per mouse (Figure-1C). Additional tumor masses were present in central venous blood vessels, the diaphragm, and lymph nodes of vector transfectants (data not shown). In contrast, with the *ILK* transfectants, only one of the two animals that developed tumors showed a single metastatic colony in the lung. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). These results clearly demonstrate that the expression of *ILK* in human MDA-MB-435 breast carcinoma cells significantly suppresses tumorigenicity and metastatic ability in athymic nude mice.

## **2. Methods:**

### **Cell migration assay**

Cell migration assays were performed as described earlier(25) using modified Boyden chambers (tissue culture-treated, 6.5 mm diameter, 10- $\mu$ m thickness, 8- $\mu$ m pores, Transwell®; Costar Corp., Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 10  $\mu$ g/ml vitronectin in PBS. The *ILK* transfected and control cells were harvested with 0.05% Trypsin-EDTA, washed twice with quenching medium (serum free medium containing 5% BSA), and then resuspended in quenching medium (10<sup>6</sup> cells/ml). About 50,000- 100,000 cells were then added to the top of each



migration chamber and allowed to migrate to the underside of the top chamber for 6 h at 37°C in a CO<sub>2</sub> incubator. The nonmigratory cells on the upper membrane were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane were washed with PBS, extracted with 300ul extraction buffer and absorbance determined at 560nm. All values have had background subtracted, which represents cell migration on membranes coated with BSA (1%). Each determination represents the average of three individual wells, and error bars represent the standard deviation (SD).

### **Analysis of Cell Surface Integrin Profiles**

Fluorescence-activated cell analysis (26) was used to identify the integrin profiles on MDA-MB-435 cells in response to *ILK* expression. Monolayer cultures (60-80% confluency) *ILK* transfected and control cells were trypsinized and washed in culture medium. Briefly, harvested cells were divided into equal aliquots of  $2.5 \times 10^5$  cells/ml in serum free medium plus 1% BSA. After two washes in this medium the cells were resuspended in 1:50 dilution of anti- $\alpha v \beta 3$  or  $\alpha 5 \beta 1$  specific antibody (Chemicon) in serum-free medium plus 1% BSA for 1h on ice. After two washes in serum-free medium plus 1% BSA, the cells were incubated in 1:100 dilution of F(ab')<sub>2</sub> secondary anti-goat antibody conjugated with FITC (ICN Biomedicals) in this same medium for 1h on ice. The cells were washed twice in PBS/ 0.1% BSA and resuspended in the same solution. These samples were then analyzed using a Becton Dickinson FACScan and the data analyzed using the CellQuest software.

### Tumorigenicity and Metastasis Assays

Cells ( $10^6$ ) were injected into the subaxillary mammary fat pads of 4-6 week-old female athymic nude mice Ncr nu/nu (10-12 mice/group; Taconic Labs, Germantown, NY) as described (27). Mice were maintained under the guidelines of NIH and the Cleveland Clinic Foundation. All protocols were approved and monitored by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumors were monitored weekly after inoculation. When the mean tumor diameter reached 1.0-1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastases.

## **C. CONCLUSIONS:**

In previous reports, we have provided evidence that *ILK* expression is down-regulated in primary breast tumors and in cell lines derived from metastatic breast tumors. We have shown that *ILK* overexpression inhibits the growth of the highly metastatic breast cancer cell line MDA-MB-435. In addition, *ILK* overexpression stimulates the levels of the growth suppressing integrin  $\alpha 5 \beta 1$  and inhibits the levels of  $\alpha v \beta 3$ , a growth promoting integrin. These innovative studies suggest a novel role for *ILK* in the etiology of breast cancer. Functional studies *in vitro* and in animal models were therefore undertaken to establish *ILK* as a metastasis suppressor gene. These studies are part of this year's report.

The present study reveals that expression of *ILK* potently suppresses *in vitro* and *in vivo* tumorigenicity of the human mammary carcinoma cells. The MDA-MB-435 cells are a model for deficient *ILK* protein expression and transfection of the *ILK* gene is designed to restore this deficiency. As shown in the last year's report, the growth suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth suppression of MDA-MB-435 cells. These results suggest a possible role for *ILK* in the suppression of tumor growth and metastasis and directly implicate its loss in processes regulating the malignant phenotype in human breast cancer. *ILK* seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary and second,

it modulates the levels of integrins, transmembrane receptors that have been shown to regulate growth, differentiation and invasiveness of cells. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes and to induce tumor growth and metastasis in nude mice (Figures-1 and 2).

**D. KEY RESEARCH ACCOMPLISHMENTS:**

- Chromosome 11 harbors a breast cancer metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the growth of the metastatic breast cancer cell line MDA-MB-435 both in vitro and in vivo.

These data suggest that ILK functions as a metastasis suppressor gene in breast cancer

**E. REPORTABLE OUTCOMES:**

- These results are being prepared as a manuscript for publication.



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**FIGURE LEGEND:**

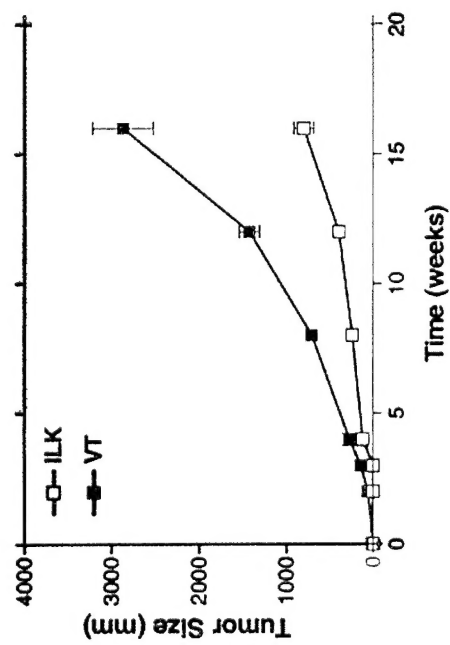
**Figure-1:** Cell invasion assay of MDA-MB-435 cells transfected with vector (VT), full length *ILK* and its variants ( $\Delta$ ANK, E359K). Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lysed and absorbance determined at 560 nm. (B) Flow cytometric analysis of  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  integrins expressed on the surface of *ILK* transfected and parental MDA-MB-435 cells. The relative fluorescence intensity of cells stained with  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  antibodies is represented as percentage of cell shift. Bars represent S.E.

**Figure-2:** (A) In vivo tumor growth of *ILK* transfected (-) and vector transfected ( ) MDA-MB-435 cells in mammary fat pads of athymic nude mice. Each point represents the mean  $\pm$  SE of tumors. (B) Five  $\times 10^5$  cells of *ILK* transfected (top panel) or vector transfected (bottom panel) MDA-MB-435 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks at which time the mice were photographed and sacrificed. (C) Lung colony formation in athymic nude mice injected with vector transfected (VT) or *ILK* transfected (*ILK*) MDA-MB-435 cells. Bars represent S.E.

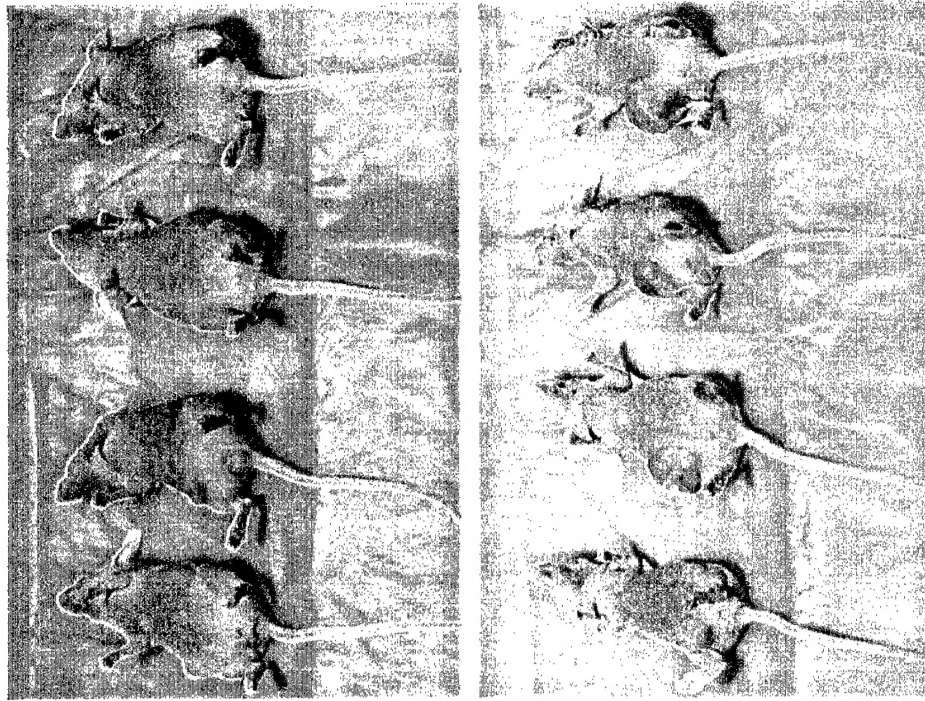
FIGURE 1



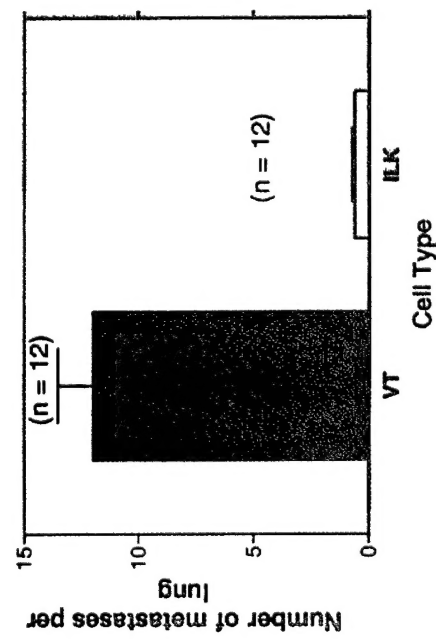
A.



B.



C.



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FIGURE 2